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Immunocontraceptive Effects in Male Rats Vaccinated with Gonadotropin-Releasing Hormone-I and -II Protein Complex

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Immunocontraception has been suggested as an optimal alternative to surgical contraception in animal species. Many immunocontraceptive vaccines have been designed to artificially produce antibodies against gonadotropin-releasing hormone-I (GnRH-I) which remove GnRH-I from the vaccinated animals. A deficiency of GnRH-I thereafter leads to a lack of gonadotropins, resulting in immunocontraception. In this study, we initially developed three immunocontraceptive vaccines composed of GnRH-I, GnRH-II, and a GnRH-I and –II (GnRH-I+II) complex, conjugated to the external domain of *Salmonella* Typhimurium flagellin. As the GnRH-I+II vaccine induced significantly ($p < 0.01$) higher levels of anti-GnRH-I antibodies than the other two vaccines, we further evaluated its immunocontraceptive effects in male rats. Mean testis weight in rats ($n = 6$) inoculated twice with the GnRH-I+II vaccine at 2-week intervals was significantly ($p < 0.01$) lower than in negative control rats at 10 weeks of age. Among the six vaccinated rats, two were non-responders whose testes were not significantly reduced when compared to those of negative control rats. Significantly smaller testis weight ($p < 0.001$), higher anti-GnRH-I antibody levels ($p < 0.001$), and lower testosterone levels ($p < 0.001$) were seen in the remaining four responders compared to the negative control rats at the end of the experiments. Furthermore, seminiferous tubule atrophy and spermatogenesis arrest were found in the testis tissues of responders. Therefore, the newly developed GnRH-I+II vaccine efficiently induced immunocontraception in male rats. This vaccine can potentially also be applied for birth control in other animal species.

Keywords: Immunocontraception, gonadotropin-releasing hormone, antibody, testosterone, spermatogenesis

Introduction

Immunocontraception is accomplished by blocking the biological roles of reproductive hormones. It is now considered as a plausible alternative to surgical sterilization to improve animal welfare [1]. Immunocontraceptive vaccines have mainly been applied to improve meat quality and reduce the aggressiveness of male domestic animals [2, 3]. However, the application of immunocontraceptive vaccines has also been extended to the control of wildlife and stray pet populations [4, 5]. Because gonadotropin-releasing hormone-I (GnRH-I) is the most important reproductive hormone, several immunocontraceptive vaccines have been developed using GnRH-I as an immunogen. GnRH-I is a highly conserved decapeptide found in almost all vertebrates and even in some invertebrates [6]. After the initial identification of GnRH-I, originally known as mammalian GnRH, several GnRH-I variants have since been found in many animal species. It is well known that GnRH-I is critically involved in the development of reproductive systems in mammals. However, the physiological roles of other variants, including GnRH-II, are largely unknown [7]. Some studies have suggested that GnRH-II may be indirectly involved in spermatogenesis [8].

GnRH-I is released from the hypothalamus and subsequently stimulates the production of follicle-stimulating

hormone (FSH) and luteinizing hormone (LH) in the pituitary [6]. FSH and LH regulate gonadal functions in both male and female animals. For instance, FSH induces spermatogenesis and LH stimulates the secretion of testosterone in male animals. Anti-GnRH-I antibodies, produced in response to the injection of a large dose of GnRH-I-contraceptive vaccine, remove GnRH-I peptides from the bloodstream. The subsequent deficiency of GnRH-I results in the decreased production of FSH and LH, which eventually leads to the suppression of spermatogenesis and therefore, contraception in male animals [1, 3].

However, the main challenge in developing these contraceptive vaccines using GnRH-I, is the low immunogenicity of the intact GnRH-I peptide. Therefore, several carrier proteins, including bacterial toxoids, have been conjugated to GnRH-I to increase its immunogenicity [9]. Bacterial flagellin is known as a strong carrier-adjuvant material that greatly enhances the immunogenicity of antigens conjugated with it [10]. In our previous study, we developed an immunocontraceptive vaccine containing six copies of GnRH-I conjugated to *Salmonella* Typhimurium flagellin (STF-2), which efficiently induced contraceptive effects in male rats. In the current study, we developed a new form of GnRH vaccine that contains both GnRH-I and –II conjugated with STF-2, to further enhance immunogenicity. The newly developed vaccine effectively inhibited spermatogenesis in the testis of male rats.

Materials and Methods

Recombinant GnRH-I, GnRH-II, and GnRH-I+II Complex Proteins

Three types of recombinant GnRH proteins were generated as follows: 12 copies of GnRH-I, 12 copies of GnRH-II, and a complex consisting of 6 copies each of GnRH-I and -II (GnRH-I+II). All recombinant proteins were conjugated to STF-2 protein (GenBank accession no. ERO08334) by a GGGS linker between its isoleucine 239 and threonine 243 residues. The synthesized genes were inserted into the pQE-40 vector (Qiagen, Germany) by cleaving at BamHI and HindIII sites with restriction enzymes (New England Biolabs, USA). The recombinant GnRH proteins were expressed in *E. coli* according to the manufacturer's procedures (Qiagen) [11, 12]. The identities of recombinant proteins were determined by western blotting with rabbit anti-GnRH (cat no. ab8491; Abcam, UK) and anti-flagellin polyclonal antibodies (cat no. ab93713, Abcam), as previously described [13−16].

Prediction of Recombinant GnRH Protein Structures

The intensive modeling mode of the Phyre version 2.0 (protein homology/analogy recognition engine) program was used to predict the structures of the recombinant GnRH-I, GnRH-II, and GnRH-I+II proteins conjugated to STF-2. The predicted structures were visualized using the 3D-mol viewer of Vector NTI Advance 9.1.0 software (Invitrogen, USA).

Immunization of Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (approval no. IACUC16135). A total of 36 specific pathogen-free, 4-week-old Sprague Dawley male rats were used in this study. Rats were purchased from a commercial animal supplier (Orient Bio, Korea). In the first experiment, 24 rats were assigned into 4 groups (negative control, GnRH-I-vaccinated, GnRH-II-vaccinated, and GnRH-I+II-vaccinated groups), with 6 rats per group. The first vaccine, containing 500 μg of recombinant GnRH protein, was intramuscularly administered into rats at week 0. A booster vaccine, containing the same amount of GnRH protein, was injected 2 weeks after the first vaccination. Rats in the negative control group were injected with PBS at the same time. Blood samples were collected from all rats at weeks 0, 2, and 4. Serum samples were used to determine anti-GnRH antibody titers. In the second experiment, a total of 12 rats were assigned into a negative control or a vaccination group, with 6 rats per group. Rats in the vaccination group were injected twice with 500 μg of the recombinant GnRH-I+II protein at weeks 0 and 2. Rats in the negative control group were administered PBS at the same time. Blood samples were collected from all rats at weeks 0, 2, 4, 6, 8, and 10. Serum samples were used to determine anti-GnRH antibody titers and testosterone concentrations. At the end of the experiment, all rats were euthanized by intramuscular injection of alphaxalone (10 mg/kg) and their testes were weighed.

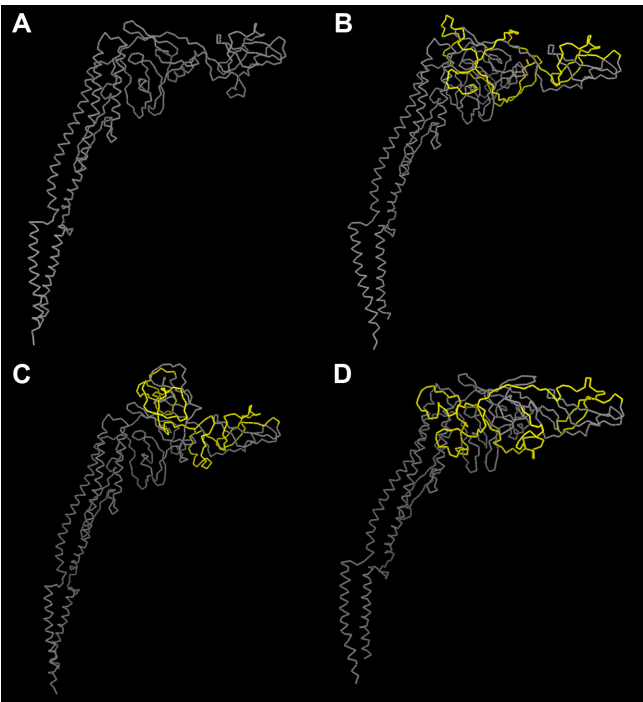### Anti-GnRH-I Antibody and Testosterone Levels

Serum samples collected from rats in the first and second experiments were used to determine their antibody titers and testosterone levels. Anti-GnRH-I antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), as previously described [13]. Testosterone concentrations were determined by an enzyme immunoassay (EIA; Neodin Vetlab, Korea), as previously reported [13].

Histological Studies

At the end of the experiments, testes and epididymides were surgically excised from all rats and weighed. Testis samples were fixed in Bouin's Solution (Sigma Aldrich, USA) and embedded in paraffin wax. Testicular tissue sections were stained with hematoxylin and eosin (HE). The level of atrophy in testis samples was determined by a pathologist at the College of Veterinary Medicine, Konkuk University.

Statistical Analysis

Data were analyzed by a one-way or two-way ANOVA, with Bonferroni correction, using GraphPad Prism v 5.00 (GraphPad Software Inc, USA).

Fig. 1. The predicted structures of (**A**) naive STF-2, (**B**) 12 copies of GnRH-I, (**C**) 12 copies of GnRH-II, and (**D**) a complex of 6 copies of GnRH-I and –II peptides inserted into the external domain of the STF-2 protein.
Gray and yellow colors indicate the STF-2 protein and 12 copies of the GnRH peptide located at the external domain of STF-2, respectively.
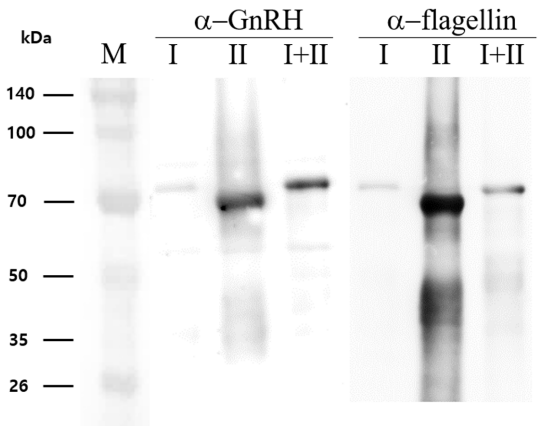
Results

Detection of Recombinant GnRH Proteins

The predicted structures of the newly designed recombinant GnRH-I, GnRH-II, and GnRH-I+II proteins conjugated to STF-2 showed that all three GnRH peptides were located at the external domain of STF-2 protein, as expected (Fig. 1). The three recombinant GnRH proteins were expressed in *E. coli* and detected by western blotting using antibodies specific to GnRH-I and STF-2 (Fig. 2). All three recombinant GnRH proteins appeared to have molecular weights of approximately 72 kDa.
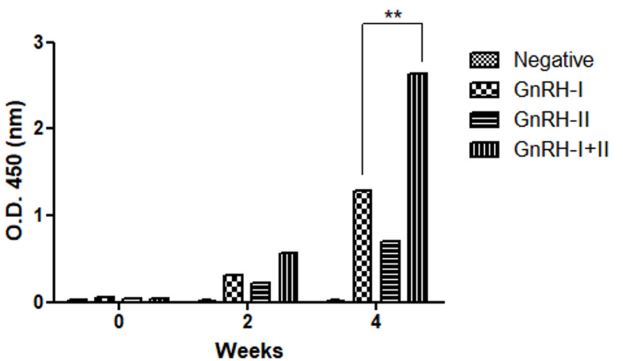
Selection of a Vaccine Candidate

Initial experiments were performed to determine which GnRH vaccine could induce the highest anti-GnRH-I antibody titers. Anti-GnRH-I antibody levels were determined by ELISA of serum samples collected from rats vaccinated with recombinant GnRH-I, GnRH-II, or GnRH-I+II proteins and from negative control rats. As expected,
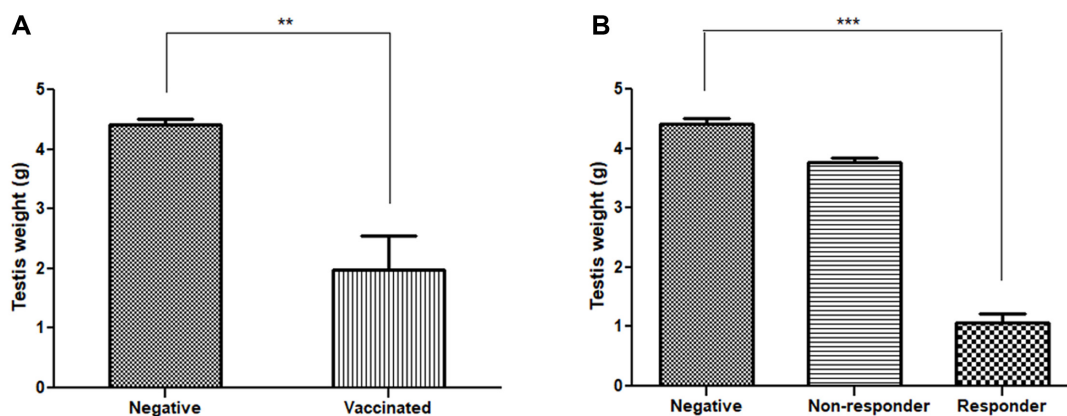

Fig. 2. Detection of recombinant GnRH proteins by western blotting.
Recombinant GnRH-I, GnRH-II, and GnRH-I+II peptides conjugated to STF-2, were detected using anti-GnRH and anti-flagellin polyclonal antibodies. The size of the recombinant GnRH proteins was calculated at approximately 72 kDa.

rats in the negative control group did not produce anti-GnRH-I antibodies. In all rats inoculated with the three different recombinant GnRH vaccines, anti-GnRH-I antibodies were detectable at 2 weeks after the first vaccination. However, robust production of antibodies was not identified until 4 weeks after the first vaccination, which was 2 weeks after receiving a booster vaccination (Fig. 3). The highest titers of anti-GnRH-I antibodies were found in rats vaccinated with the GnRH-I+II complex. The GnRH-I+II vaccine resulted in significantly higher titers than the GnRH-I or GnRH-II vaccines ($p < 0.01$). Anti-GnRH-I antibodies were identified in rats vaccinated with

Fig. 3. Anti-GnRH-I antibody titers in serum samples obtained from negative control rats and rats vaccinated with GnRH-I, GnRH-II, or a GnRH-I+II complex.
Statistical significance was determined by two-way ANOVA, with Bonferroni correction, at **$p < 0.01$. Data are presented as mean ± SEM.

Fig. 4. Testis weights of negative control rats and rats vaccinated with a GnRH-I+II complex.
Statistical significance was determined by one-way ANOVA, with Bonferroni correction, at **$p < 0.01$ and ***$p < 0.001$. Data are presented as mean ± SEM.

GnRH-II, but their titers were much lower than those found in rats vaccinated with GnRH-I (Fig. 3). Therefore, we selected the GnRH-I+II vaccine for subsequent studies evaluating contraceptive effects in male rats.

Testis Weights

The testes of 6 rats were weighed 10 weeks after the first vaccination with the GnRH-I+II protein complex. Mean testis weight was compared with that of negative control rats. Testes from rats vaccinated with GnRH-I+II weighed significantly less ($p < 0.01$) than testes from the negative control rats (Fig. 4A). Vaccinated rats showed a 55.5% reduction in testis weight compared to negative control rats (1.97 g vs 4.42 g). However, a significant reduction in testis weight was not seen in two of the six vaccinated rats. These rats are hereafter referred to as "non-responders" and the remaining four, as "responders." The average testis weight of the four responders was significantly lower ($p < 0.001$, 75.9% reduction) than that of the negative control rats, whereas it was not significantly reduced in the two non-responders (14.5% reduction, Fig. 4B).
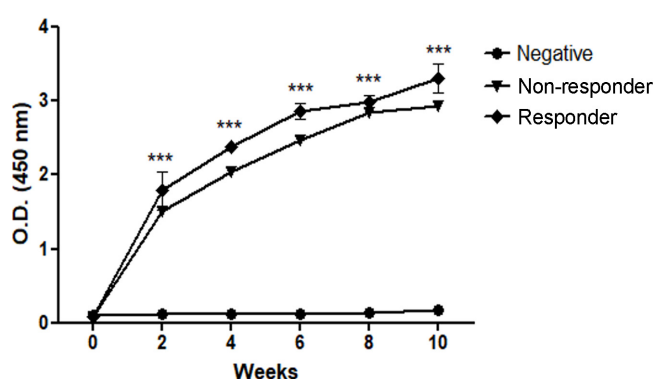
Anti-GnRH-I Antibodies

Anti-GnRH-I antibody titers were determined by ELISA in serum samples collected from negative control and vaccinated rats. As expected, anti-GnRH-I antibodies were not detected in negative control rats. However, anti-GnRH-I antibody titers were significantly higher ($p < 0.001$) in both responder and non-responder rats vaccinated with the GnRH-I+II complex than in negative control rats during an 8-week experimental period (from 2 to 10 weeks, Fig. 5).

The first vaccination at week 0 had already induced significantly higher antibody titers after 2 weeks ($p < 0.001$). The second vaccination at week 2 boosted the production of antibodies until the end of the study. During the experimental period, the antibody titers of non-responders were slightly lower than those of responders, but this was not statistically significant.
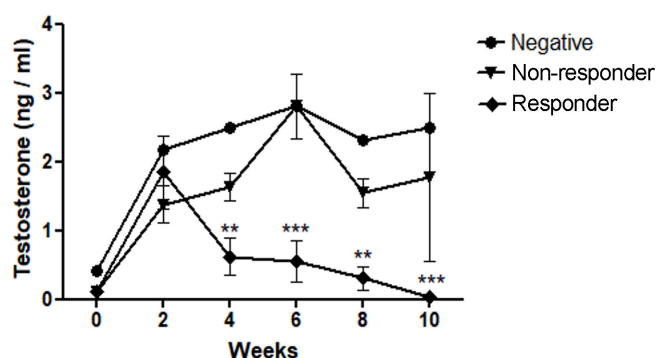
Testosterone Concentrations

Serum testosterone concentration was determined by EIA in negative control and vaccinated rats. Serum testosterone levels in rats of the negative control were maintained at the range of 2.17–2.82 ng/ml from weeks 2 to 10. The

Fig. 5. Anti-GnRH-I antibody titers in serum samples from negative control rats and rats vaccinated with a GnRH-I+II complex.
Statistical significance was determined by two-way ANOVA, with Bonferroni correction, at ***$p < 0.001$. Data are presented as mean ± SEM.

Fig. 6. Testosterone levels in serum samples from negative control rats and rats vaccinated with a GnRH-I+II complex. Statistical significance was determined by two-way ANOVA, with Bonferroni correction, at **$p < 0.01$ and ***$p < 0.001$. Data are presented as mean ± SEM.
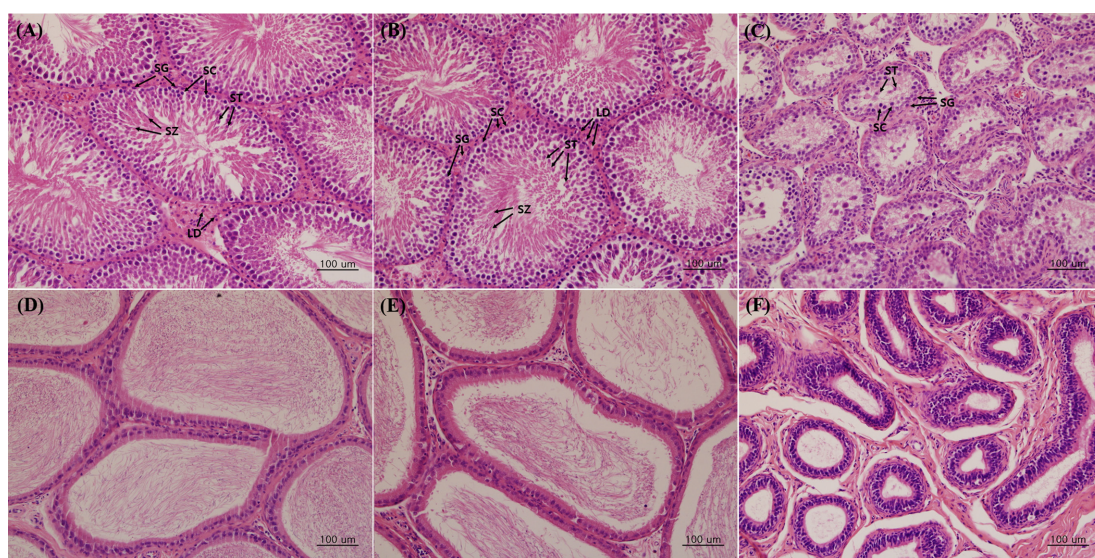
testosterone levels in responders were similar to those of negative control rats 2 weeks after the first vaccination. However, their testosterone levels significantly ($p < 0.01$) decreased from week 4 (2 weeks after the second vaccination; 0.61 ng/mL) to week 10 (0.025 ng/ml, Fig. 6). These testosterone levels were 24.7% and 1.0% of the levels in the negative control group at 4 and 10 weeks, respectively. Testosterone levels in the non-responders were not significantly different than those of the negative control rats during the entire experimental period (Fig. 6).

Histological Analysis

Testis tissues of negative control rats showed fully developed seminiferous tubules, with spermatogonia, spermatocytes, spermatids, and spermatozoa displaying normal morphology (Fig. 7A). Testis tissue from non-responders appeared to be very similar to testis tissue from negative control rats (Fig. 7B). In addition, severe atrophy of the seminiferous tubules was identified and spermatozoa were not detected in the responder rats (Fig. 7C). Normal development of the epididymis duct was found in negative control and non-responder rats (Figs. 7D and 7E). However, the epididymis duct was identified in responder rats (Fig. 7F).

Discussion

GnRH-I is highly conserved across mammalian species. It is secreted from the hypothalamus and induces the maturation of reproductive systems, through the production of LH and FSH in the pituitary gland in both male and female animals [6]. Therefore, several types of immuno-contraceptive vaccines have been developed, using GnRH-I as an antigen, to produce anti-GnRH-I antibodies in male domestic animals, experimental animals, and pets [17-21]. Unlike GnRH-I, GnRH-II expression has only been identified in a few animal species, including humans and pigs [2]. Interestingly, GnRH-II is expressed in many organs, including in male and female reproductive systems

Fig. 7. Histological evaluation of testes and epididymides.
(**A**) Normal testis development in a negative control rat, (**B**) normal testis development in a non-responder, (**C**) severe testicular atrophy in a responder, (**D**) normal epididymis duct in a negative control rat, (**E**) normal epididymis duct in a non-responder, and (**F**) severe atrophy of epididymis duct in a responder. SG: spermatogonia, SC: spermatocyte, ST: spermatid, SZ: spermatozoa, LD: Leydig cells.

of humans and animal species [2]. However, the exact role of GnRH-II is still not fully understood. It has recently been suggested that GnRH-II may be indirectly involved in spermatogenesis [8].

In this study, we initially developed three types of immunocontraceptive vaccines composed of recombinant GnRH-I, GnRH-II, or a GnRH-I+II complex, linked to the external domain of STF-2. Because bacterial flagellin, a ligand of toll-like receptor 5, has strong adjuvant effects, it is often used as a carrier of low-immunogenic peptides such as GnRH or proteins to increase immune responses [10, 22, 23]. We evaluated the efficacies of these vaccines for the production of anti-GnRH-I antibodies. Among the immunocontraceptive vaccines tested, the GnRH-I+II vaccine produced the highest anti-GnRH-I antibody titer in male rats. When recombinant GnRH-II protein alone was injected into rats, the anti-GnRH-II antibodies that were produced showed a weak cross-reactivity with GnRH-I protein. However, when the recombinant GnRH-I+II protein complex was administered to rats, anti-GnRH-I antibody titers were significantly higher than those produced by the injection of GnRH-I or GnRH-II proteins alone. These results suggested that GnRH-II may synergistically induce the production of anti-GnRH-I antibodies in vaccinated rats. This kind of synergism may be attributed to the conserved amino acid sequences at the N-terminus and C-terminus of GnRH-I and –II [2, 6].

The testes of rats (n = 6) vaccinated with the GnRH-I+II vaccine weighed significantly less than those of negative control rats. However, two (33%) of the six vaccinated rats did not respond to the vaccine. Although the testis weights of these non-responders were slightly reduced, they were not statistically different from the testis weights of the negative control rats. Non-responders are frequently identified in several animal species, including rats, after treatment with several types of GnRH-I-based immuno-contraceptive vaccines [21, 24–26]. Approximately 20–40% of rats and 33% of cats vaccinated with immunocontraceptive vaccines have been shown to be non-responders [21, 24, 25]. Similar results were found in this study. The lack of response in some animals vaccinated with immuno-contraceptive vaccines may be partially explained by unusual immune responses, such as the existence of suppressor T cells, but the exact mechanism is still unknown [19, 27]. In this study, we designed the immunocontraceptive vaccine to place the GnRH-I and -II complex at the external domain of STF-2 using a computer program. However, we did not confirm the recombinant protein structure produced from *E. coli* by nuclear

magnetic resonance spectroscopy. If the protein structure was altered during the expression or purification process, it might cause low immunogenicity and lead to relatively low vaccine efficacy. This anticipated concern should be checked in a future study. Most non-responder animals have lower levels of anti-GnRH-I antibodies, but higher concentrations of testosterone than the responders [21, 24, 25]. In our study, similar patterns of antibody and testosterone were observed from the non-responders, but the differences were not statistically significant. The lower levels of testosterone in responders would lead to atrophy of their testes [21, 24, 25]. Histological analysis shows inhibition of spermatogenesis in the testis tissues of responders [19, 21]. Our study also demonstrated suppression of spermatogenesis, with lower numbers of spermatogonia, spermatocytes, spermatid, and spermatozoa in the testis of responders. New immunoconatraceptive vaccines formulated with different doses of GnRH proteins, more potent adjuvants, or different carrier proteins might be applied to animals on the expectation of improved contraceptive effects in further studies.

In conclusion, the immunocontraceptive vaccine containing both GnRH-I and -II peptides linked to STF-2, induced anti-fertility effects by suppressing testicular function in male rats. We expect that this vaccine can potentially be applied to pets, as an alternative to surgical contraception methods.

Acknowledgments

Conflicts of Interest

The authors have no financial conflicts of interest to declare.

References

1. Delves PJ. 2004. How far from a hormone-based contraceptive vaccine? *J. Reprod. Immunol.* **62:** 69-78.
2. Desaulniers AT, Cederberg RA, Lents CA, White BR. 2017. Expression and role of gonadotropin-releasing hormone 2 and its receptor in mammals. *Front. Endocrinol.* **8:** 269.

3. Thompson Jr DL. 2000. Immunization against GnRH in male species (comparative aspects). *Anim. Reprod. Sci.* **60:** 459-469.

4. Kirkpatrick JF, Lyda RO, Frank KM. 2011. Contraceptive vaccines for wildlife: a review. *Am. J. Reprod. Immunol.* **66:** 40-50.

5. Levy JK. 2011. Contraceptive vaccines for the humane control of community cat populations. *Am. J. Reprod. Immunol.* **66:** 63-70.

6. Kochman K. 2012. Evolution of gonadotropin-releasing hormone (GnRH) structure and its receptor. *J. Anim. Feed Sci.* **21:** 3-30.

7. Kauffman A. 2004. Emerging functions of gonadotropin-releasing hormone II in mammalian physiology and behaviour. *J. Neuroendocrinol.* **16:** 794-806.

8. Khan MA, Prevost M, Waterston MM, Harvey MJ, Ferro VA. 2007. Effect of immunisation against gonadotrophin releasing hormone isoforms (mammalian GnRH-I, chicken GnRH-II and lamprey GnRH-III) on murine spermatogenesis. *Vaccine* **25:** 2051-2063.

9. Earl ER, Waterston MM, Aughey E, Harvey MJ, Matschke C, Colston A, *et al.* 2006. Evaluation of two GnRH-I based vaccine formulations on the testes function of entire Suffolk cross ram lambs. *Vaccine* **24:** 3172-3183.

10. Qian F, Guo A, Li M, Liu W, Pan Z, Jiang L, *et al.* 2015. Salmonella flagellin is a potent carrier–adjuvant for peptide conjugate to induce peptide-specific antibody response in mice. *Vaccine* **33:** 2038-2044.

11. Hu W, Xiang J-Y, Kong P, Liu L, Xie Q, Xiang H. 2017. Expression and characterization of a single-chain variable fragment against human LOX-1 in *Escherichia coli* and brevibacillus choshinensis. *J. Microbiol. Biotechnol.* **27:** 965-974.

12. Choi GS, Kim HJ, Kim EJ, Lee SJ, Lee Y, Ahn J-H. 2018. Stepwise synthesis of quercetin bisglycosides using engineered *Escherichia coli. J. Microbiol. Biotechnol.* **28:** 1859-1864.

13. Song Y, Kim D, Nam H, Lee J, Park S, Song C, *et al.* 2012. Evaluation of the efficacy of immunocastration vaccine composed of gonadotrophin-releasing hormone conjugated with *Salmonella typhimurium* flagellin in rats. *Reprod. Domest. Anim.* **47:** e47-e50.

14. Kim E, Myoung J. 2018. Hepatitis E virus papain-like cysteine protease inhibits type I interferon induction by down-regulating melanoma differentiation-associated gene 5. *J. Microbiol. Biotechnol.* **28:** 1908-1915.

15. Kang S, Choi C, Choi I, Han K-N, Roh SW, Choi J, *et al.* 2018. Hepatitis E virus methyltransferase inhibits type I interferon induction by targeting RIG-I. *J. Microbiol. Biotechnol.* **28:** 1554-1562.

16. Park C, Kim Y-H, Lee S-R, Park S, Jung Y, Lee Y, *et al.* 2018. Characterization of recombinant bovine sperm hyaluronidase and identification of an important Asn-X-Ser/Thr motif for its activity. *J. Microbiol. Biotechnol.* **28:** 1547-1553.

17. Cook R, Popp J, Kastelic J, Robbins S, Harland R. 2000. The effects of active immunization against GnRH on testicular development, feedlot performance, and carcass characteristics of beef bulls. *J. Anim. Sci.* **78:** 2778-2783.

18. Dunshea F, Colantoni C, Howard K, McCauley I, Jackson P, Long K, *et al.* 2001. Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. *J. Anim. Sci.* **79:** 2524-2535.

19. Ferro V, Khan M, McAdam D, Colston A, Aughey E, Mullen A, *et al.* 2004. Efficacy of an anti-fertility vaccine based on mammalian gonadotrophin releasing hormone (GnRH-I)—a histological comparison in male animals. *Vet. Immunol. Immunopathol.* **101:** 73-86.

20. Han X, Gu L, Xia C, Feng J, Cao X, Du X, *et al.* 2015. Effect of immunization against GnRH on hypothalamic and testicular function in rams. *Theriogenology* **83:** 642-649.

21. Levy JK, Miller LA, Crawford PC, Ritchey JW, Ross MK, Fagerstone KA. 2004. GnRH immunocontraception of male cats. *Theriogenology* **62:** 1116-1130.

22. Cuadros C, Lopez-Hernandez FJ, Dominguez AL, McClelland M, Lustgarten J. 2004. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. *Infect. Immun.* **72:** 2810-2816.

23. Turley CB, Rupp RE, Johnson C, Taylor DN, Wolfson J, Tussey L, *et al.* 2011. Safety and immunogenicity of a recombinant M2e–flagellin influenza vaccine (STF2. 4xM2e) in healthy adults. *Vaccine* **29:** 5145-5152.

24. Han X-F, Li J-L, Zhou Y-Q, Ren X-H, Liu G-C, Cao X-H, *et al.* 2016. Active immunization with GnRH-tandem-dimer peptide in young male rats reduces serum reproductive hormone concentrations, testicular development and spermatogenesis. *Asian J. Androl.* **18:** 485-491.

25. Yao Z, Si W, Tian W, Ye J, Zhu R, Li X, *et al.* 2018. Effect of active immunization using a novel GnRH vaccine on reproductive function in rats. *Theriogenology* **111:** 1-8.

26. Zeng XY, Turkstra JA, Meloen RH, Liu XY, Chen FQ, Schaaper WM, *et al.* 2002. Active immunization against gonadotrophin-releasing hormone in Chinese male pigs: effects of dose on antibody titer, hormone levels and sexual development. *Anim. Reprod. Sci.* **70:** 223-233.

27. Sad S, Gupta H, Talwar G, Raghupathy R. 1991. Hyporesponsiveness to a GnRH vaccine in a non-responder mouse strain is T-cell mediated. *J. Reprod. Immunol.* **20:** 189-194.